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The Biochemical Cytology of Liver (Abstract)*

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A better understanding of hepatic physiology and pathology will result from application of the techniques of biochemical cytology: electron microscopy, cytochemical staining of cells *in situ*, analysis of subcellular fractions isolated from tissue homogenates by differential centrifugation, autoradiography, and the localization of fluorescent antibody in tissue sections.

In our laboratory we have employed the first three of these techniques in a study of the rat liver cell. The fine structure of this cell, as revealed by electron microscopy, has been summarized elsewhere[1, 2]. Electron microscopy has emphasized the extent to which the structure of mitochondria may be preserved in the process of homogenization and centrifugation[1-3]. The "microsomes" sedimented from this tissue consist largely of fragments of "ergastoplasm" or basophilic material. In the cell, this is a series of inter-connected cytoplasm membranes (endoplasmic reticulum) on the surface of which are arranged the ribonucleoprotein particles of Palade. The endoplasmic reticulum is continuous with the outer of the two nuclear membranes and, at the other end, it may be continuous with the plasma membrane.

From the biochemical study of the isolated fractions has come a vast body of information which emphasizes the complexity and inter-relatedness of the subcellular units[3]. For example, oxidative phosphorylation occurs in the mitochondria. It utilizes DPN**, apparently synthesized in the nucleus, and yields ATP. The latter is utilized in a great many processes, including the initial step in protein synthesis, amino acid activation. This reaction is catalyzed by enzymes recovered in the "supernatant fluid" which remains unsedimented even with high-speed centrifugation Then, in the ribonucleoprotein particles of Palade, the activated amino acids are incorporated into proteins.

There are probably other aspects of dependence of cytoplasmic protein synthesis upon the nucleus. Both chemical evidence and some morphologic observations are consistent with the view that cytoplasmic ribonucleoprotein is derived, at least in part, from nuclear ribonucleoprotein. Nuclear ribonucleoprotein, localized in either nucleolus or chromatin, thus appears to be an important means of transmitting information from genic DNA to the cytoplasm.

Electron microscopy has demonstrated that the plasma membrane surface is in-

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** Abbreviations: DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; ATP-ase, adenosine triphosphatase; DNA, desoxyribose nucleic acid; 3'MeDAB, 3'methyl, dimethylaminoazobenzene.

creased enormously in wide areas of the hepatic cell. These are the absorptive surfaces exposed to the sinusoids (space of Disse) and the secretory surfaces exposed to the bile canaliculi[4-6]. It has been possible to localize two important phosphatases in these microvilli by combining electron microscopy with the staining methods of Wachstein and Meisel[7]. Essner and associates[8] showed that the microvilli of the canaliculus surfaces have high levels of ATP-ase and 5'-nucleotidase activities, whereas in those of the sinusoidal surfaces, 5'-nucleotidase, but not ATP-ase, is demonstrable. This enzymatic differentiation of the secretory and absorptive surfaces of the cell probably has important physiological significance. Alterations in such enzymes may be anticipated in disease states. It may be relevant that we find a polarized mitochondrial orientation in the rat liver cell similar to that described by Pollister[9] in *Amphiuma*. Most mitochondria are parallel to a line between sinusoid and canaliculus.

On either side of the bile canaliculi there are cytoplasmic structures the inter-relationships of which still remain to be established. These are the Golgi apparatus, acid-phosphatase-rich granules and "dense bodies".

The multiple nature of the Golgi apparatus is readily revealed by the classical silver or osmium techniques. Careful study shows them always to be situated adjacent to the bile canaliculi[10]. Electron microscopy[4, 6] demonstrates the Golgi bodies of this tissue to have the typical fine structure: parallel membranes and associated vacuoles. (For general reviews of different aspects of the Golgi apparatus see references 11 to 15.)

In 1947 Deane[16] noted that the peribiliary acid phosphatase granules had shapes suggestive of the Golgi apparatus. In work still in progress, we find that under three experimental conditions (bile duct ligation, carbon tetrachloride poisoning, and ingestion of the carcinogen, 3'MeDAB) the Golgi apparatus and acid-phosphatase granules change their intracellular location in a similar manner. However, only with the advent of electron microscopy has resolving power been sufficiently high and the nature of the Golgi apparatus sufficiently defined to permit critical determination of the relation of the acid-phosphatase granules, Golgi apparatus, and other peribiliary structures.

It would be of great importance to establish whether acid phosphatase activity is localized in the peribiliary "dense bodies". In work with Beaufay and de Duve we[17] found such bodies, first described in tissue sections by Rouiller[18], to be present in isolated subcellular fractions which were rich in acid phosphatase activity. It was suggested that perhaps these bodies were the *lysosomes* postulated by de Duve and others[19].

Since the most purified lysosome fractions studied by us contained many mitochondria, it was not possible to establish the peribiliary dense bodies as the sites of acid phosphatase activity[17]. We are currently using electron microscopy in an attempt to localize the enzyme in thin sections of fixed liver incubated in the acid phosphatase medium. In this fashion we hope to clarify the relationship of acid phosphatase granules, dense bodies and Golgi apparatus.

The lysosome concept may be of great importance to pathologists. If de Duve is correct, the hydrolytic enzymes of the cell are contained in small cytoplasmic granules, lysosomes, surrounded by membranes impermeable to the substrates which the enzymes can hydrolyze. Cell autolysis would involve access of substrate and enzyme, through lysosome rupture or more subtle alteration of permeability.

The biochemical cytology of liver must emphasize the quantitative differences among the cells, dependent upon their position in the hepatic lobule. Every cytological structure and enzyme activity which has been studied shows this lobular heterogeneity. There is more ATP-ase and alkaline phosphatase activity in the bile canaliculi of the peripheral zone than in those of the centrolobular zone. On the other hand, 5'-nucleotidase activity is higher in the canaliculi (and peribiliary granules) of the centrolobular cells. The Golgi apparatus is larger and acid phosphatase activity higher in the peripheral cells. Of the microsomal enzymes, glucose-6-phosphatase is very active in the peripheral cells; in the centrolobular

cells it is lower and more variable. Esterase activity is high throughout the lobule, with the highest activity in the centrolobular zone. Lipase and non-specific choline esterase activities are also higher in the centrolobular cells. The mitochondria in the cells of the peripheral zone are long, stout filaments. They become finer as the cells near the central vein are approached. In the latter cells the mitochondria are spherical, fewer in number and very weakly stained in the Baker acid-haematin stain for phosphatide. Correlated with this is the succinic dehydrogenase activity demonstrable by the tetrazolium staining technique; it is very low in the cells adjacent to the central vein and high in the peripheral cells of the lobule. On the other hand, DPNH-Cytochrome C reductase activity as well as the activities of the DPN-linked lactic and beta-hydroxybutyric dehydrogenases, are high throughout the lobule but more so in the centrolobular cells.

It is possible to offer tentative suggestions for the functional significance of some of these quantitative differences. Thus, the area of high glucose-6-phosphatase activity is that in which glycogen is first deposited and first mobilized. The area of highest lipase and esterase activity is that in which lipid first appears, in a variety of experimental conditions. This is also where pigments like lipofuscin are first seen. Finally, the low succinic dehydrogenase activity of the centrolobular cells may be related to the low oxygen tension of the blood entering the central vein. If the visualized succinic dehydrogenase activity reflects the level of aerobic respiration (Krebs cycle) and the visualized lactic dehydrogenase activity the level of glycolysis, this would mean that the centrolobular cells derive a greater proportion of their energy from glycolysis than from aerobic respiration. Such heterogeneity of enzyme distribution may be involved in the selective localization, in the hepatic lobule, of lesions due to poisons, toxins and infectious agents.

The cell changes familiar to pathologists are beginning to be re-examined with the newer techniques under discussion. Thus, cloudy swelling in a variety of circumstances appears to involve a rounding and enlargement of the mitochondria in which the inner membranes undergo profound change[20].

Recently we studied a liver biopsy from a 72 year old man, made available to us by Dr. Joseph Ehrlich. The fine structures of nucleus, mitochondria, ergastoplasm and bile canaliculi are essentially similar in human and rat liver. The same is true for the staining of the bile canaliculi for ATP-ase, 5'-nucleotidase, and alkaline phosphatase; of the peribiliary granules for acid phosphatase; and of the mitochondria for succinic dehydrogenase, DNP-linked and TPN-linked dehydrogenases and associated cytochrome C reductases. We shall mention but one difference, to indicate that species differences do exist and to suggest its possible clinicopathologic interest. In human liver, unlike that of the rat, there is intense alkaline phosphatase activity on the sinusoidal aspects of the cells. Indeed, there is much more activity there than in the bile canaliculi, and perhaps more attention should be paid to it in human liver disease.

By studying cells with varying amount of lipofuscin we have tentatively arrived at a description of the changes which may occur in the hepatic cell. The first lipofuscin granules are formed adjacent to the bile canaliculi; their appearance coincides with the disappearance of the peribiliary acid phosphatase granules. It is of interest that Pfuhl[21] suggested, on the basis of the peribiliary location of the granules, that lipofuscin appears within the Golgi bodies.

As lipofuscin droplets become more frequent they are also found further from the canaliculi. When the droplets become very numerous, it seems that the ATP-ase and 5'-nucleotidase activities disappear from the canaliculi and the canaliculi themselves can no longer be seen. There is, however, no diminution in the activities of the dehydrogenases studied (succinic, lactic, beta-hydroxybutyric) and of DPNH-cytochrome C reductases, until perhaps the very end in some cells which may be dying. Nor is there any apparent diminution of sinusoidal enzyme activity.

In electron micrographs (of tissue fixed in osmium tetroxide) the lipofuscin granules consist of a homogeneous electron-dense material arranged around irregular

vacuoles. Few, or even single, vacuoles are noted in bodies about the size of mitochondria, or somewhat larger. In the large lipofuscin droplets there are a great many vacuoles of various shapes. Large spherical droplets, presumably lipid, are frequently found in the same cells. These have thin irregular electron-dense coating, and a small inner granular core. The largest part appears empty, suggesting extraction of material during processing of the tissue. Perhaps the outer layer corresponds to lipofuscin.

Further studies, with animals on experimental regimes as well as with human material, will be required to establish any relations that may exist between lipofuscin granules and either mitochondria or the Golgi apparatus.

It has been our purpose to convey a general picture of the biochemical cytology of the liver and to suggest the value of its techniques to problems of pathology and aging.

Mechanism of Hepatic Fibrosis

(Abstract)

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The important aspects of hepatic fibrosis are its types, histogenesis, histochemical and chemical nature, and its functional significance. These were studied in human biopsy and autopsy material and in animals experimentally intoxicated with ethionine, carbon tetrachloride or thioacetamide or placed on low protein/high fat diets.

Fibrosis may be solely in the portal tract, in the periportal or centrolobular zones, anywhere within the lobule, and focal. These types of fibrosis do not disturb the lobular architecture in contrast to cirrhosis characterized by regenerative nodules and connective tissue septa. The pathways by which cirrhosis develops are: 1) collapse following disappearance of liver cells (postnecrotic cirrhosis), 2) septa formation most often associated with fatty metamorphosis but also with chronic passive congestion, granulomas and hemochromatosis (septal cirrhosis), and 3) inflammation and scarring around bile ducts and ductules either primary or secondary to extrahepatic biliary obstruction (biliary cirrhosis). The three basic pathways do not each imply a specific etiology and are associated with several types of fiber formation.

Basic observations in other organs apply to collapse and fiber formation in the presence of fibroblasts in the liver. A particular problem is fiber formation around fatty or damaged liver cells and around ductular cells. For this it was advantageous to reexamine the distribution of fibers in the normal liver, particularly in sections one micron thick (studies with F. Paronetto and H. van der Noen).

The border between the normal liver cell and the sinusoid is marked by an amorphous and partly fibrillar layer which gives the periodic acid Schiff reaction after removal of glycogen by diastase (DPAS) and a few small DPAS positive granules are in the Kupffer cells. Chromotrope aniline blue (CAB) stains the border zone hazy purple with fine blue reinforcements which can be impregnated with silver. This reticulum appears in thin sections as a row of points or dashes indicating cross or tangentially cut fibers. Around ductules (cholangioles) a continuous black line is seen in silver stains

indicating a basement membrane which also gives DPAS reaction and is blue in CAB stain.

Around damaged liver cells in hepatitis or cirrhosis, a thicker amorphous layer is purple in CAB stains. Frequently a few irregular DPAS positive granules are found in the liver cells and denser granules which vary greatly in size are in the Kupffer cells. On the base of the liver cells a continuous DPAS positive line appears while in silver impregnations a dense row of points or dashes suggests increase in reticulum fibers or formation of a basement membrane similar to that of ductular cells. Around fatty liver cells such a structure is also noted which is sometimes even duplicated and in extreme cases gives a collagen reaction. Fibroblasts are not conspicuous. In rats with ethionine intoxication producing single cell necrosis and hepatocellular degeneration, DPAS positive granules in liver cells and Kupffer cells and the amorphous and fibrillar material on the base of the liver cell are very conspicuous. In addition, cells with oval- or spindle-shaped nuclei accumulate between the liver cell plates (oval cells of Farber). They are occasionally arranged as ductules and a fine canalicular system between these cells can be visualized by injecting the bile ducts with India ink. This indicates their epithelial origin despite their frequently mesenchymal appearance. Around such ductular cells, much DPAS-positive amorphous and fibrillar material can be seen. Silver impregnates fibers or membranes which may be multilayered even in thin sections. Some of the fibers and membranes in contact with the ductular cells continue away from them into the surrounding tissue. Fibroblasts are again not demonstrable. This relation of fiber formation to ductular cells is also noted in man, e.g., in cirrhosis and in biliary obstruction.

The fibrillar material, impregnated by silver with a positive DPAS and blue CAB reaction, becomes metachromatic after sulfation and is not digested by trypsin or hyaluronidase. It is therefore reticulin and transitions into collagen which are observed in silver impregnation and van Gieson stains. The amorphous substance on the sinusoidal border of the liver cells and the granules in the damaged liver cells and proliferating Kupffer cells which give a DPAS reaction and are purple with CAB become also metachromatic on sulfation and partially react with colloidal iron and Alcian blue. The material is digested by trypsin but not by hyaluronidase, pectinase or ribonuclease, and it loses its PAS reaction on acetylation. It is apparently a polysaccharide protein complex which may be a matrix for the formation of scleroprotein fibers, collagen and reticulum. The question is left open as to whether the similarity in the histochemical reaction of the perisinusoidal material and the granules in the Kupffer cells indicates that the latter engulf the former, acting as scavenger cells, or whether the cells form it and thus assume the function of fibroblasts. The formation of fibers in this matrix around the damaged liver cells and ductular cells suggests that these structures play an inducing role in determining the site and extent of the fibrosis. Since the ductular cells are possibly derived from liver cells, dedifferentiation may be the common factor in stimulating the fibrosis.

Further support is obtained in recent experiments (carried out by F. G. Zak, F. Paronetto and I. Bubelis) with carrageenin, a polysaccharide derived from moss. Within two days after its injection into the liver, a necrotic area is surrounded by a ground substance giving acid mucopolysaccharide reaction in which fibroblasts seem to develop from adventitial cells of arteries and capillaries and from Kupffer cells. The ductular cells also proliferate profusely and sometimes ductular cells cannot be distinguished from fibroblasts. Abundant reticulum is formed particularly in approximation to the ductular cells although some fibers continue away from them. The violent fibroblastic reaction which completely subsides in two weeks illustrates the rapid formation of ground substance and reticulum in the vicinity of fibroblasts and ductular cells.

To supplement the histochemical studies, the amino acid, hydroxyproline, which occurs almost exclusively in collagen, was determined in dried and defatted liver tissue and in a fraction extracted by O.1 N NaOH believed to contain procollagen (in-

vestigations of F. Hutterer and E. J. Singer in human cirrhosis). The increase of collagen is associated with a relatively greater increase of the alkaline soluble fraction. Serial experiments indicated that the hepatic hydroxyproline and hexosamine content rises precipitously to three times the normal during the seven weeks on an ethionine diet. Much of this is in the NaOH soluble fraction and coincides with a marked increase of the reticulum histologically. A rise in proline in the alkaline soluble fraction only precedes the rise in hydroxyproline, suggesting a precursor relationship. Therefore, during active fibrosis, polysaccharide appears around liver cells while procollagen and then collagen is formed, utilizing hepatic proline which is converted to hydroxyproline.

In assessing the functional significance of hepatic fibrosis, the interposition of a mucoprotein or scleroprotein material between sinusoids and liver cells is thought to interfere with some hepatic functions such as the free exchange of metabolites between the liver and blood. The interruption of bile flow through such a connective tissue accumulation is demonstrated in primary or secondary biliary cirrhosis in which the obstructive features parallel the development of a thick fibrotic layer. The connective tissue septa in cirrhosis carry anastomotic vessels between the hepatic artery, portal vein and hepatic veins, shunting blood from the hepatic parenchyma and transmitting arterial pressure into the portal system, thereby creating many of the cardinal symptoms of cirrhosis.

In conclusion, hepatic fibrosis poses the same problems as accumulation of fibrous tissue elsewhere, modified by the determining role of altered epithelial elements and by the consequences that are related to the functions of the liver and its double blood supply.

DISCUSSION

MAX WACHSTEIN: I really think that this is a memorable meeting, since it must bring to everyone's mind that pathology is now in a period of dynamic transformation. It is about 100 years since Virchow introduced the concept of cellular pathology which has remained the basic foundation of our discipline. During the last decades, the impact of this concept has lost much of its scientific drive. However, within the last years, new techniques have been developed to such a degree that they now can be used for the study of normal and diseased tissues. Just to name a few: spectrophotometry, fluorescent microscopy, histochemistry, phase microscopy, electron microscopy and others. With the aid of these new techniques, cellular pathology can now be explored in terms of functional pathology and the study of cells can be extended to subcellular and even molecular levels.

It is impossible to discuss the wealth of material presented today. Just to comment briefly on one point made by Dr. Novikoff: dehydrogenases were first determined in tissue extracts by biochemists. The great improvement in histochemical techniques both with regard to fixation as well as to the reagents used enables one to localize specific dehydrogenase, as Dr. Novikoff has shown, in various portions of the liver lobules; DPN mediated dehydrogenases, for instance, are found mainly around central and succinic dehydrogenase around periportal fields. In addition to the topographical localization, these stains demonstrate the mitochondria as sites of enzymatic activity. Most recently Pearse and Scarpelli[22] have even been able to identify specific areas within isolated mitochondria as sites of dehydrogenase activity. It seems perfectly feasible that changes in enzymatic components of mitochondria may characterize various disease states which cannot be distinguished at present with the usual routine techniques. To take another example: if one considers the difficulty of proper diagnosis of the various forms of liver disease, one cannot help but be hopeful that the application of the electron microscope, with or without various enzyme staining techniques, will become very helpful in their understanding and differentiation. Cholestasis, for instance, occurs in many abnormal conditions. It seems obvious that the study of the bile canaliculi with the electron microscope, aided possibly by the adenosine tri-

phosphate technique, will be of help in the understanding of the cause for this abnormality.

As regards Dr. Popper's presentation, I think it is very important that he stressed the fact that fibrosis in the liver does not always originate in the fibroblasts. It is difficult to comment, in a short discussion, on the variety of histologic and biochemical techniques he has used. I would, however, like to make the following specific point: in the rats given ethionine—he has not mentioned that he, himself, first described the interesting ethionine changes in the liver—we have consistently found fairly extensive areas of central necrosis which he also mentioned in one of his papers[23]. These central areas of necrosis later tend to extend into periportal fields and it is sometimes difficult to say from where they started. Similar areas of atrophy and necrosis have been described by Opie in rats fed butter yellow[24]. In these areas, fibroblastic proliferation was described and has also been shown by Morrione[25] to occur in animals treated by repeated inhalations of carbon tetrachloride or fed butter yellow. It would thus appear that the total amount of hydroxyproline measured chemically may also be derived from collagen produced by typical fibroblasts.

I thought I might contribute most to this discussion by showing a few pertinent slides. In a section of a normal human liver biopsy, the bile canaliculi stained very well with the adenosine triphosphatase technique[7]. When liver biopsies of cases of jaundice were studied, one could see how the bile canaliculi extended toward the sinusoids thus making it perfectly understandable that in biliary obstruction reflux from the bile into the liver may occur[26]. In this connection, I would like to ask Dr. Novikoff whether in his electron micrographs, the Disse spaces can be made out as consistent structures, which separate the bile canaliculi from the sinusoids, as has been suggested by Rouiller[6].

Enzymatic staining reactions in ethionine-induced liver damage reveal interesting changes. The ductular cells which Dr. Popper has discussed show a very marked depression of enzymatic activity as compared to normal liver cells. In contrast, regenerating liver lobules show a very strong reaction for various enzymes. If ethionine administration is continued long enough, hepatomas will occur. In such hepatomas alkaline phosphatase activity showed a very strong reaction[27].

In concluding this discussion, I should like to say that all of us who are practicing hospital pathologists are most grateful for these presentations which so clearly reveal the areas into which scientific pathology is now moving. It is quite obvious that hospital pathologists cannot do all of this type of work; we do not have the equipment and often not the time, but nevertheless hospital pathologists like myself can work in any of the new fields that have been opened by these new techniques. The possibilities are endless and the reward is the satisfaction of participating in this exciting phase of scientific pathology.

HANS POPPER: The presence of fibroblasts in the rat liver after ethionine intoxication is a problem that has puzzled us for a considerable time. Variation of ethionine diets produces a great variety of hepatic injuries and, while some procedures produce unquestionable central necrosis, the animals of the series studied chemically in our laboratory for developing fibrosis failed to reveal such necrosis. In these specimens no typical fibroblasts were found. However, I would like to emphasize that Dr. MacDonald of the Boston City Hospital has seen beautiful fibroblasts in rat cirrhosis induced by low choline/high fat/low protein diets, and we have seen them in some instances of ethionine cirrhosis. In the latter case, the question arose as to whether these were actually fibroblasts or modified ductular cells, particularly since we could inject between these cells channels from the biliary system. This problem is also illuminated by transitions between ductular cells and fibroblasts seen particularly well in carrageenin granulomas within livers of rats treated with ethionine. I am therefore not able to answer your question because I am not quite sure any more what a fibroblast really is and how to differentiate it occasionally from cells of apparently epithelial origin.

ALEX B. NOVIKOFF: In the present stage of development of electron microscopy it would be foolhardy to suppose that "seeing is believing". We would not categorically assert that artefacts are not involved in the many discontinuities seen in the cells lining the sinusoids or the spaces seen between the lining cells and the hepatic cell surfaces. However, it seems unlikely that shrinkage or other artefacts would produce such well-structured microvilli as are found on the cell surfaces in that region. These are like the microvilli found in many tissues where cells are exposed to cavities. It seems reasonable to suppose: 1) that the lining cells do not completely separate the liver cells from the sinusoids, and 2) that the microvilli project into a plasma-filled space, the Disse space. Just as the excretory surface at the bile canaliculus is increased by microvilli so is the absorptive surface at the Disse space. The two surfaces are probably differentiated enzymatically, as our findings with phosphatases indicate.

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